# Sepharose® CL

## For gel filtration and affinity chromatography

# Contents

Chemical and physical properties ... 3
  Stability in aqueous media ... 5
  Stability in organic solvents ... 5
  Thermal stability ... 6

Applications ... 6
  Gel filtration ... 6
    Gel filtration of agar at 50°C ... 6
    Gel filtration of cellulose in cadoxen ... 7
    Gel filtration in 6 M guanidine hydrochloride ... 7
    Gel filtration in organic solvents ... 9

  Affinity chromatography ... 9
    Activation with cyanogen bromide ... 9
    Coupling in organic solvents ... 10
    O-alkylations ... 11
    O-acylations ... 11
    Isolation of antibodies to blood group substance A by affinity chromatography ... 11

Experimental methods ... 12
  Preparation of the gel for use in aqueous media ... 12
  Preparation of the gel for use in organic solvents ... 12
  Gel filtration in aqueous media ... 13
  Gel filtration in organic solvents ... 14
  Gel filtration at elevated temperatures ... 14
  Affinity chromatography ... 15
  Cleaning ... 15
  Storage ... 15

Availability ... 15

References ... 16

# Sepharose CL-2 B, 4B and 6 B

The bead-formed agarose gel Sepharose® has long been recognized as being an excellent medium for affinity chromatography and gel filtration studies of proteins, polysaccharides, nucleic acids and other high molecular weight substances. Now Pharmacia Fine Chemicals is pleased to introduce an outstanding new chromatographic medium Sepharose CL.

Sepharose CL is prepared from Sepharose by covalent cross-linkage of the individual agarose chains. Because of its greatly increased stability you can

- Perform gel filtration studies in strongly denaturing media in the range pH 3—14
- Prepare derivatives in organic solvents for affinity chromatography
- Use organic solvents for gel filtration
- Sterilize it repeatedly by autoclaving
- Use Sepharose CL as a matrix for affinity chromatography when harsh elution conditions are to be used
- Obtain excellent flow rates
- Carry out chromatography at temperatures up to 70°C

# Chemical and physical properties

Sepharose CL is prepared from Sepharose by reaction with 2,3-dibromopropanol under strongly alkaline conditions.* This produces a cross-linked agarose gel with substantially the same porosity as the parent gel, but with greatly increased thermal and chemical stability. After cross-linking, the gel is desulphated by alkaline hydrolysis under reducing conditions (1). The reactions involved and a partial structure of Sepharose CL are shown in Figure 1 and 2. It should be noted that the resultant cross-links have the same structure as those present in Sephadex® (2) and in the epichlorohydrin cross-linked agarose gels described by *Porath, Jansson* and *Låås* (1).

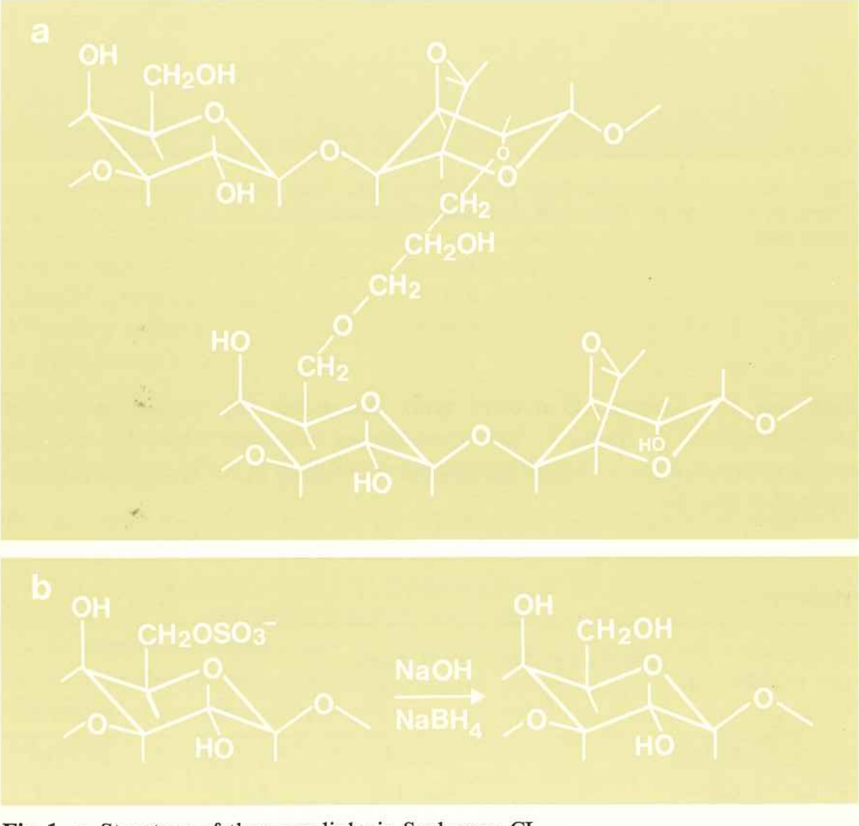

**Fig. 1.** a. Structure of the cross-links in Sepharose CL.
b. Loss of sulphate groups by alkaline hydrolysis under reducing conditions (1).

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* Patented 1971. UK Patent 1 352 613

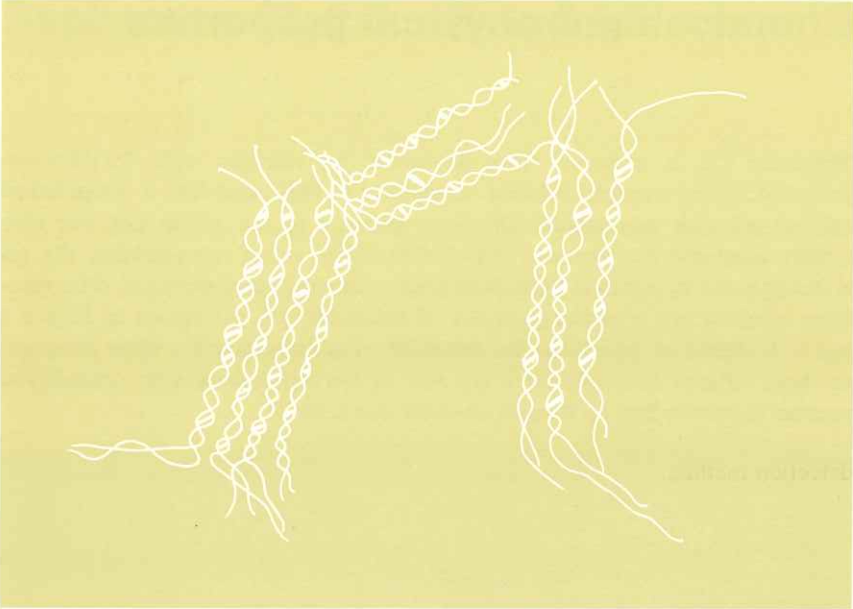

**Fig. 2.** Position of the cross-links in Sepharose CL. Note that cross-links involve the chains in a single gel fibre and that inter-fibre cross-links do not occur to a significant degree.

Sepharose CL is prepared in three types. Some relevant physical properties are summarized in Table 1. The approximate exclusion limits for proteins and dextrans in aqueous buffer solutions are the same as for the corresponding types of Sepharose.

**Table 1**

| Type | Approx.agarose conc. % | Bead diam. wet $\mu$m | Approx. exclusion limits daltons | |
|---|---|---|---|---|
| | | | Polysaccharides | Proteins |
| Sepharose CL-2B | 2 | 60—250 | $20\times10^6$ | $40\times10^6$ |
| Sepharose CL-4B | 4 | 40—190 | $5\times10^6$ | $20\times10^6$ |
| Sepharose CL-6B | 6 | 40—210 | $1\times10^6$ | $4\times10^6$ |

# Stability in aqueous media

Sepharose CL can be used in aqueous media in the range pH 3—14. Its stability in alkaline media is particularly high (1). Under oxidizing conditions, limited hydrolysis of the polysaccharide chains may occur.

The data in Table 2 demonstrate the stability of Sepharose CL-4B in solutions of chaotropic ions. Sepharose CL-4B was packed in a Pharmacia column K 16/40 (bed volume 57.4 ml) and equilibrated with sodium phosphate buffer (0.02 M) containing NaCl (0.15 M). One bed volume of the same buffer containing the chaotropic salt KSCN (3 M) was passed through the column and the carbohydrate content of the effluent was estimated by the anthrone reaction. The column was allowed to stand overnight in the KSCN solution and the determination was repeated. After the initial elution, the concentration of carbohydrate in the effluent was below the lower limit of sensitivity of the detection method.

**Table 2**
Stability of Sepharose CL in presence of 3 M KSCN

| Sample | Carbohydrate concentration $\mu$g/ml |
|---|---|
| First effluent | 6.5 |
| After 24 h exposure | $<0.5$ |
| After 48 h exposure | $<0.5$ |
| After 72 h exposure | $<0.5$ |

# Stability in organic solvents

The gel structure of agarose differs significantly from that of Sephadex in that the gel-forming fibres are relatively stiff bundles of polysaccharide chains and not flexible single chains (3). Replacement of the water in the gel with other solvents has therefore a relatively small effect on pore size. Sepharose CL can be transferred from water to other solvents by the method described in the experimental section (see pages 12—13). Amongst solvents tested in our laboratories are ethanol, dimethylformamide, tetrahydrofuran, acetone, dimethyl sulphoxide, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50). Examples of organic syntheses and gel filtration studies carried out with Sepharose CL in organic solvents are shown on pages 9—11.

# Thermal stability

Sepharose CL may be repeatedly sterilized by autoclaving at pH 7, 110—120°C. No significant changes in porosity or flow rate were found after autoclaving for 11 hours under these conditions.

# Applications

## Gel filtration

Sepharose CL is ideal for gel filtration studies of very large molecules. Its stability to a wide variety of dissociating and denaturing media makes it particularly valuable for the study of high molecular weight substances which are insoluble or aggregated in ordinary aqueous buffer systems. The great stability of Sepharose CL to alkaline conditions makes it easy to clean the gel if material should precipitate in the gel bed. Adsorption of basic proteins is greatly reduced by desulphation after cross-linking. The examples below show how Sepharose CL can deal with the most difficult elution systems.

### Gel filtration of agar at 50°C

Sepharose CL-4B has been used to study the effects of reduction on the molecular weight distribution of New Zealand agar (4). To prevent the sample from gelling in the column, the whole separation has to be carried out at a temperature above the melting point of the agar. Sepharose CL-4B was packed in a Pharmacia column K 16/100 and equilibrated with sodium phosphate buffer (0.05 M, pH 7.0) at 50°C. The void volume was determined with a sample of tobacco mosaic virus. The samples were the original agar and agar which had been treated with NaOH (5 M, 0.3% $NaBH_4$, 2 hours, 80°C) and subsequently reduced with $LiAlH_4$ in dioxane. Figure 3 shows the elution curves obtained. Carbohydrate was estimated by the orcinol method and virus was estimated by monitoring at 280 nm (4).

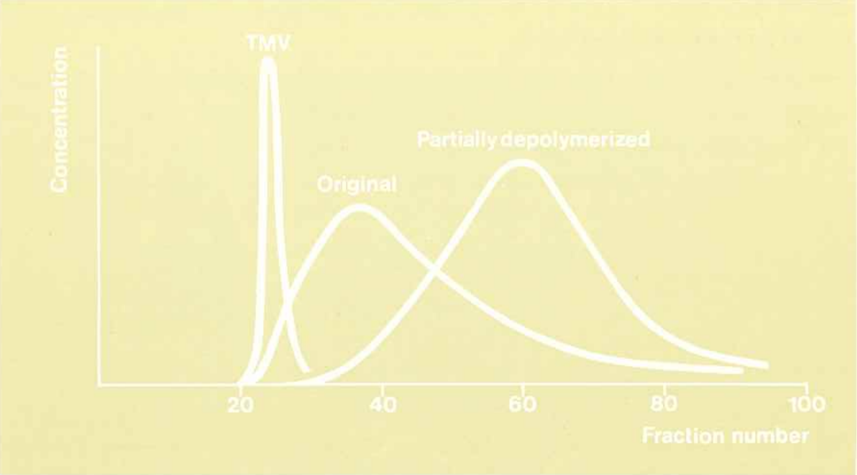

**Fig. 3.** Gel filtration of agar on Sepharose CL-4B at 50°C. Pharmacia column K 16/100; bed height 88.5 cm; eluent, sodium phosphate buffer (0.05 M, pH 7.0); flow rate, 7.9 ml/hour; sample, agar solution (0.5 %, 1 ml); fraction size, 2 ml. Tobacco mosaic virus was used to mark the void volume. (T. Låås, personal communication.)

## Gel filtration of cellulose in cadoxen

Molecular weight distribution analysis of cellulose by gel filtration has been complicated by the fact that solvents for cellulose are usually incompatible with the chromatographic media (5). Sepharose CL-2B can be used successfully with cadoxen, tri (ethylene diamine) cadmium hydroxide, for analysis of high molecular weight celluloses (fig. 4). Sepharose CL-2B was packed in a Pharmacia column K 16/70 and equilibrated with cadoxen/water (1:1). Cellulose was dissolved in cadoxen and, after dilution with water, samples of 1 ml were chromatographed. Carbohydrate concentrations were estimated by the orcinol method. The elution profiles obtained with cellulose from cotton linters (degree of polymerization approx. 3000) and cellulose (DP 200) are shown in Figure 4. In a separate experiment the leakage of carbohydrate from Sepharose CL-2B was investigated and found to be below the lower limit of sensitivity of the detection method employed.

## Gel filtration in 6 M guanidine hydrochloride

The use of gel filtration with Sepharose 6B in 6 M guanidine hydrochloride (6) is well established as a method for molecular weight estimations of proteins and polypeptides. However, the structure-breaking effect of guanidine hydrochloride leads to flow rates which may be inconveniently low. The greater stability of Sepharose CL-6B enables higher flow rates to be used in this and similar techniques. Figure 5 shows flow rates obtained with Sepharose CL-6B in 6 M guanidine hydrochloride.

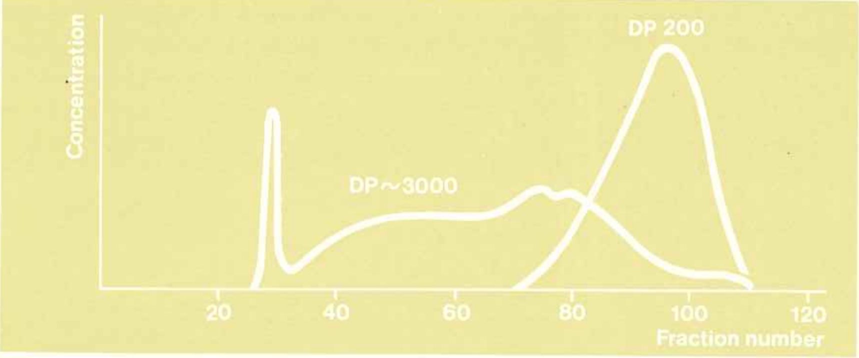

**Fig. 4.** Gel filtration of cellulose on Sepharose CL-2B in cadoxen. Pharmacia column K 16/70; bed height, 55 cm; eluent, cadoxen/water (1:1); flow rate 3.7 ml/hour; sample, cellulose solution (0.5 %, 1 ml); fraction size, 1.85 ml. (Work from Pharmacia Fine Chemicals.)

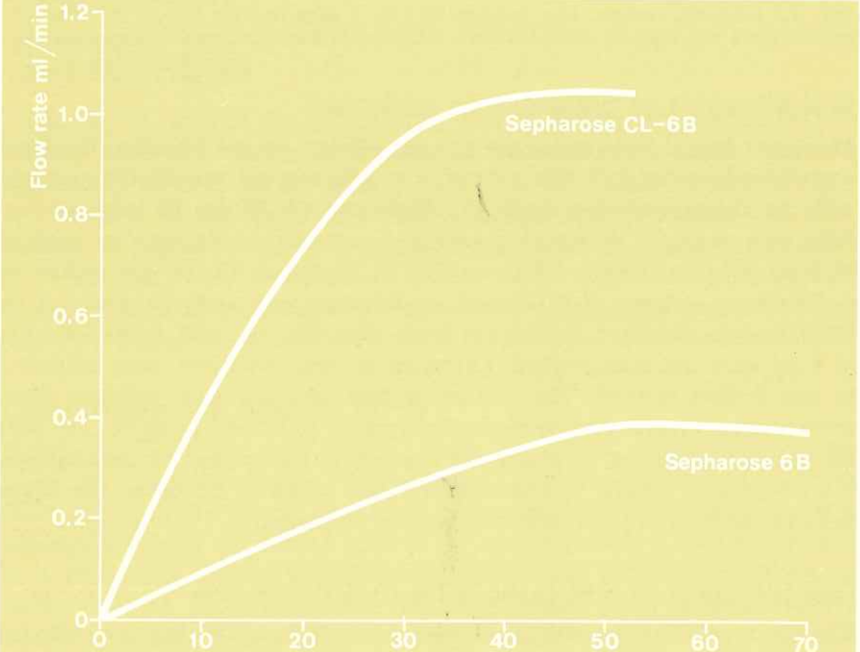

**Fig. 5.** Flow rates obtained with Sepharose CL-6B and Sepharose 6B in 6 M guanidine hydrochloride. Flow rates were measured in Pharmacia columns K 16/20 packed to a bed height of 12.5 cm.

## Gel filtration in organic solvents

Sepharose CL provides a new medium for gel filtration of large molecules in organic solvents and should prove of particular value in studies of hydrophobic proteins which are difficult or impossible to dissolve in ordinary aqueous media. The separation of two standard polystyrene samples in chloroform is shown in Figure 6. For details of how to transfer Sepharose CL from water to organic solvents, please refer to the experimental section on pages 12—13.

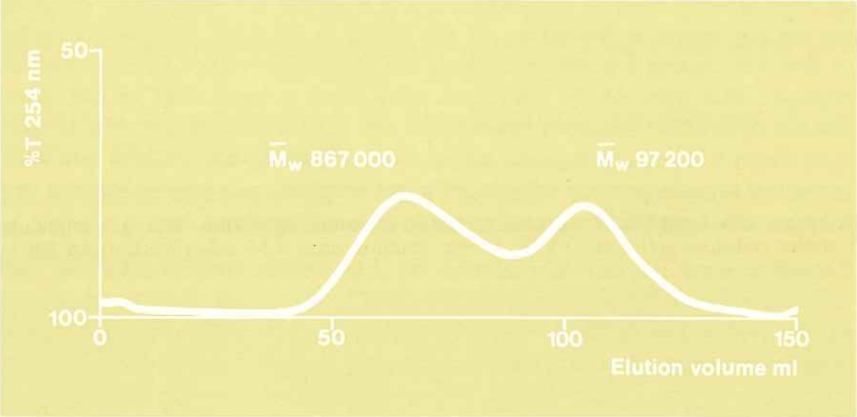

**Fig. 6.** Gel filtration of polystyrenes on Sepharose CL-4B in chloroform. Pharmacia column SR 25/45; bed height, 24.4 cm; eluent, chloroform; flow rate, 1.1 ml/min; sample, polystyrenes $\overline{M}_W$ 867,000 and $\overline{M}_W$ 97,200 in chloroform (6 mg of each in 4 ml eluent). (Work from Pharmacia Fine Chemicals.)

# Affinity chromatography

Affinity chromatography, biospecific adsorption chromatography, has quickly become one of the most useful separation methods in biochemistry. Its success is due not only to the provision of suitable methods for immobilizing ligands (7), but also to the availability of a suitable matrix, Sepharose (8). Sepharose CL with its extremely low content of sulphate groups and stability in strongly dissociating media complements Sepharose for affinity chromatography under extreme conditions.

## Activation with cyanogen bromide

Sepharose CL can be activated with CNBr in the same way as Sepharose. However, work in our laboratories has shown that, for a given CNBr to gel ratio, activation of Sepharose CL is only about 50% as efficient as activation of Sepharose. This is easily understood when it is realized that the cross-linking reaction reduces the number of hydroxyl groups which can be activated. For most applications CNBr-activated Sepharose 4B is to be recommended.

## Coupling in organic solvents

In all but a few special case the preparation of bed materials for affinity chromatography involves the preparation of a special bed material by coupling the desired ligand to Sepharose. If the ligand is insoluble in aqueous media or does not contain a substituent which can be readily coupled by conventional methods, this stage of the procedure may cause a great deal of difficulty. Considerable effort has been expended in the past in preparing special derivatives which will react in aqueous media. Since Sepharose CL is stable in a wide variety of organic solvents, simple, efficient reactions can now be carried out to prepare bed materials with water-insoluble small ligands.

Extensive work in our laboratories (9) has shown that O-acylation and O-alkylation reactions between Sepharose CL and small model compounds can be used to give products with excellent chromatographic properties. Some of these reactions are summarized in Figure 7.

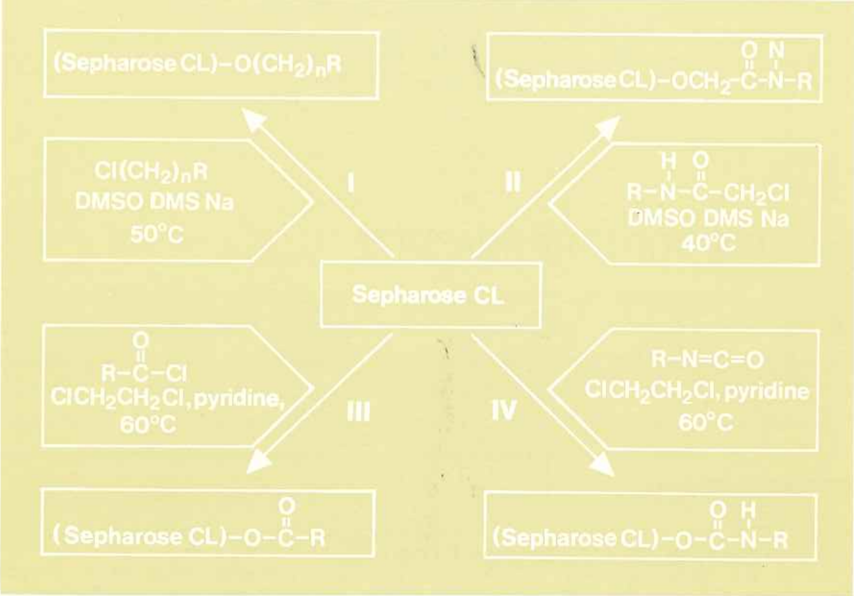

**Fig. 7.** Derivatives of Sepharose CL prepared in organic solvents.

## O-alkylations (reactions I and II)

Base-catalyzed O-alkylations provide a number of new routes to derivatives of Sepharose CL. Reactions I and II are examples of the *Hakomori* reaction (10) in which the methylsulphinyl carbanion is used as the base.

## O-acylation (reactions III and IV)

Esterification of Sepharose CL, e.g. reaction III, can be carried out directly in dichloroethane using pyridine as the base. Reaction IV is a well-known reaction previously described for dextran by *Burchard* and *Pfannemüller* (11).

## Isolation of antibodies to blood group substance A by affinity chromatography

The method of *Kristiansen* (12) was used to isolate antibodies to blood group on Sepharose 2B. After partial acetylation, substance A (25 mg) was coupled to Sepharose CL-4B.

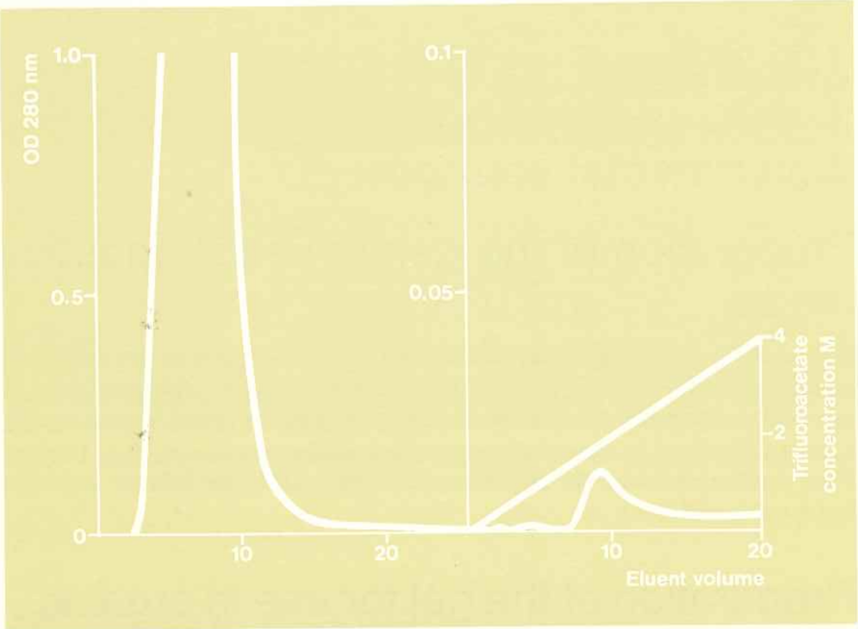

**Fig. 8.** Isolation of antibodies to blood group substance A by affinity chromatography. Bed material, blood group substance A coupled to Sepharose CL-4B; Pharmacia column K 16/20; bed height, 2.5 cm; starting eluent, phosphate buffer solution (0.02 M, pH 7.5) containing NaCl (0.15 M) followed by a linear gradient of sodium trifluoroacetate (0—4 M) in starting eluent; flow rate, 10 ml/hour; sample, anti-A immune serum (2 ml). (Work from Pharmacia Fine Chemicals.)

Blood group substance A was purified from ovarian cyst fluid by gel filtration on Sepharose 2B. After partial acetylation, substance A (25 mg) was coupled to Sepharose CL-4B (25 ml) by the CNBr method (0.5 M $NaHCO_3$, 20°C, overnight). The product was washed alternately at pH 7.5 and pH 4.5, with 4M sodium trifluoroacetate and finally with the starting buffer (0.02 M phosphate, 0.15 M NaCl, pH 7.5). The substance A — Sepharose CL-4B was packed in a Pharmacia column K 16/20 (bed height 2.5 cm) and a sample of anti-A immune serum (2 ml) diluted with starting buffer (2 ml) was applied. After elution of unadsorbed protein with starting buffer, antibodies to substance A were eluted with reversed flow using a linear gradient of sodium trifluoroacetate (0—4 M) in starting buffer (fig. 8). The initial yield of antibody activity was 69% of that applied; a further 19% could be eluted by repeating the elution sequence after 48 hours. The total yield of blood group substance A-specific antibody activity was thus 88%.

# Experimental methods

## Preparation of the gel for use in aqueous media

Sepharose CL is supplied ready swollen as a thick suspension in distilled water containing 0.02% sodium azide as a bacteriostatic agent. The suspension is too thick to be poured into a chromatographic column and it should first be diluted with eluent to a consistency which allows the easy escape of air bubbles. Final equilibration with the eluent can be carried out in the column whilst the bed is being stabilized.

## Preparation of the gel for use in organic solvents

The aqueous medium in which Sepharose CL is supplied can be exchanged for a variety of organic solvents. To ensure efficient replacement of the water by the required solvent, the transfer must be made through a graded series of solvent mixtures (13). Thus to transfer Sepharose CL from one pure solvent (A) to another pure solvent (B), the gel is transferred first to 70% A/30% B then to 30% A/70% B and finally to pure B. If A and B are not mutually miscible, the transfer is made via an intermediate solvent e.g. Sepharose CL is transferred from water to chloroform via acetone (fig. 9).

Transfer the required amount of Sepharose CL to a sintered glass Büchner funnel and remove the excess aqueous medium by gentle suction. Add the next solvent e.g. ethanol (see fig. 9) and resuspend the gel by gentle stirring. Suck off the excess solvent and resuspend in the same solvent. Repeat the process with the next solvent of the series, allowing two resuspensions, and proceed until the required solvent composition is reached. It should be noted that certain strongly dissociating media, e.g. DMSO, may cause Sepharose CL to soften. These solvents can be used in synthetic work although they may be unsuitable as eluents for gel filtration purposes.

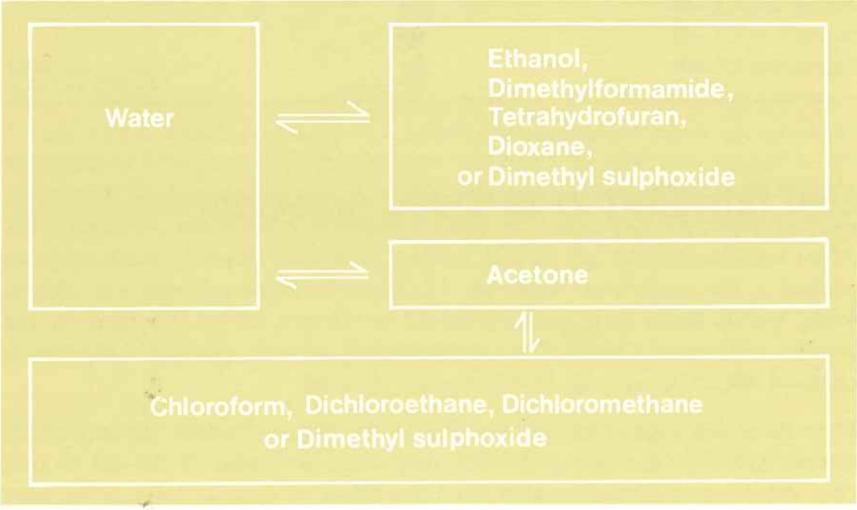

**Fig. 9.** Suggested routes for transferring Sepharose CL to organic solvents. The transfer to dimethyl sulphoxide is made most efficiently via acetone.

# Gel filtration in aqueous media

Sepharose CL may be packed in the same way as Sephadex. Full details of experimental methods for packing, sample preparation and sample application are given in the handbook "Sephadex. Gel filtration in theory and practice" which is free on request. Pharmacia columns and other chromatographic equipment have been specially designed for chromatography of biologically active substances and are recommended for reproducible results.

During packing and elution the maximum operating pressures given in Table 3 should not be exceeded. In ordinary aqueous media, flow rates obtained with Sepharose CL will be comparable to those obtained with Sepharose. In dissociating media, e.g. 6 M guanidine hydrochloride, flow rates will be higher than those obtainable with the corresponding type of Sepharose. As in all types of chromatography, the highest resolution will be obtained at low flow rates.

**Table 3**

| Type | Maximum operating pressure cm $H_2O$ |
|---|---|
| Sepharose CL-2B | 30 |
| Sepharose CL-4B | 60 |
| Sepharose CL-6B | 90 |

# Gel filtration in organic solvents

After transferring the gel to the solvent of choice, Sepharose CL may be packed in the usual way. Sepharose CL floats in dense solvents, e.g. chloroform, and in these cases packing should be carried out as described in the booklet "Sephadex LH-20. Chromatography in organic solvents". Pharmacia columns SR 25/45 and SR 25/100 should be used.

Flow rates will depend on the viscosity of the eluent. Certain solvents which disrupt hydrogen bonds, e.g. DMSO, may cause softening of the gel so that flow rates become impracticably low. The effect of organic solvents on pore size is believed to be small. Work in our laboratories has shown that the original pore size is regained when Sepharose CL is transferred from organic solvents back to aqueous media.

# Gel filtration at elevated temperatures

Gel filtration at temperatures above room temperature should be carried out in jacketed columns. Pharmacia columns K 16 and K 26 can be used at temperatures up to 60°C. The gel suspension should be warmed to the desired temperature before packing and eluents should be thoroughly degassed.

## Affinity chromatography

Methods for affinity chromatography are described in the handbook "Affinity Chromatography. Principles and methods" which is free on request.

## Cleaning

The very low content of charged groups and the inert nature of the polysaccharide chains mean that protein adsorption to Sepharose CL is very low. However, high molecular weight materials may precipitate in the column during a separation. Protein precipitates may be removed by treating Sepharose CL with sodium hydroxide solution (2 M, 2 hours) without affecting the properties of the gel. Detergents and organic solvents may be used as appropriate.

## Storage

Sepharose CL should be stored as a suspension. Sepharose CL is exceptionally resistant to biological degradation, but microbial growth may occur in the suspension medium particularly in the presence of phosphate ions. Sodium azide (0.02%), chloroform, trichlorobutanol or butanol (0.5%) may be used to prevent contamination.

## Availability

Sepharose CL is supplied in packs of 1 litre as a suspension in distilled water containing 0.02% sodium azide. It has been sterilized with diethyl pyrocarbonate.

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